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(54) Method of immobilizing and orienting molecules by use of metal chelating moieties to facilitate atomic force microscopy of proteins and molecular scale manipulation.

(57) An IgG_1 was engineered for specific adherence to a metal-containing surface by the incorporation C-terminal metal-chelating peptide (CP) and was bound to a nickel-coated mica surface. The metal-chelating peptide intereaction enabled the imaging of the proteinusing atomic force microscopy (AFM). The homogeneous appearance and calculated dimensions of the CP derivatized protein demonstrated the regiospecific orientation and stability imparted by this peptide. The ability to stably orient biomolecules to a surface may be used advantageously in a variety of processes designed to either visualize or manipulate molecular entities.

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EUROPEAN SEARCH REPORT

Application Number
EP 93 30 5866

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	ENZYME MICROB. TECHNOL., vol. 6, May 1984 pages 228-232, J.M.S. CABRAL ET AL. 'compositions and compositional-behavioural relationships of enzymes immobilized on porous inorganic supports via titanium(IV) species.' ---		G01N33/68 G01N33/92 C12Q1/68
D,A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 15, 25 May 1988 pages 7211-7215, M. C. SMITH ET AL. 'chelating peptide-immobilized metal ion affinity chromatography' ---		
D,A	US-A-4 569 794 (SMITH MICHELE C ET AL) 11 February 1986 ---		
P,X	EP-A-0 505 151 (LILLY CO ELI) 23 September 1992 * claims 9,18 * ---	1-13	
P,X	EP-A-0 497 585 (ANDERSON LESLIE DERIEMER ;COOK JAMES ALLEN (US); DAVID GARY SAMUEL) 5 August 1992 * the whole document * ---	1-13	
P,X	BIOPHYSICAL JOURNAL, vol. 64, no. 3, 10 March 1993 pages 919-924, C.R. ILL ET AL. 'A COOH-terminal peptide confers regiospecific orientiation and facilitates atomic force microscopy of an IgG1. ' * the whole document * -----	1-4,7-13	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)
C07K
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	11 July 1995	Cartagena y Abella,P

CATEGORY OF CITED DOCUMENTS
X : particularly relevant if taken alone
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PATENT ABSTRACTS OF JAPAN

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(54) METHOD FOR TRANSFER-DEPOSITING PARTICULATE FILM

(57)Abstract:
PURPOSE: To provide the method with which a particulate film is smoothly and homogeneously transfer-deposited on a solid secondary substrate.
CONSTITUTION: In this method, the surface of a solid secondary substrate is activated by irradiating it with an energy beam or subjected to treatment for providing it with hydrophobicity to form a hydrophobic surface. Alternatively, a particulate film is transfer-deposited on the surface of the solid secondary substrate by providing a specific combined ligand film on the surface of the substrate, adsorbing thiol groups on the surface of the suite to form a denatured protein or irradiating superfine particulates with an energy beam to form active radicals.

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(71)Applicant : RES DEV CORP OF JAPAN
(72)Inventor : KUNITAKE TOYOKI
ARIGA KATSUHIKO
YUURI RUBUOFU

(54) PRODUCTION OF ULTRATHIN PROTEIN FILM THROUGH ALTERNATING ADSORPTION

(57)Abstract:
PURPOSE: To obtain an ultrathin multilayered protein film having a regulated molecular structure by immersing a solid substrate alternately in a protein solution and a solution of organic polymer ions having the charge opposite to that of the protein.
CONSTITUTION: A solid substrate having a (fixed) charge on the surface is immersed alternately in two solutions; one is a protein solution (a) and the other is a solution (b) of organic polymer ions having the charge opposite to that of the protein. Immersion in the solution (b) gives a pliable thin film of the organic polymer ions on the substrate, while the subsequent immersion in the solution (a) gives a thin protein film on that film. The alternating immersion in the two solutions is repeatedly conducted and terminated with the deposition of a film of the organic polymer ions as the outermost layer.

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Two-dimensional molecular packing of proteins

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Abstract

A technique involving the physical and/or chemical adsorption of molecules in the subphase to the interfacial Langmuir monolayer has been developed to arrange protein molecules such as ferritin, bacteriorhodopsin (BR) (purple membrane) and cytochrome b_{562} in closely-packed two dimensional arrays. For better packing of molecules, site-directed mutagenesis of cytochrome b_{562} was introduced to enhance the hydrophobicity or to stabilize the molecules by introducing a cystine linkage. The photoreaction cycle of BR was analysed. From the sequence analysis of newly isolated BR-like pigments, it was suggested that several amino acid residues around the retinal were conserved and formed a "retinal pocket".

1. Introduction

In the science and technology of organic thin films the key issues are (1) the selection of materials, (2) the techniques to arrange organic molecules in a two-dimensional regular array, (3) the characterization of their ordered state, (4) the fundamental understandings of their physical properties and (5) the designated application to devices. These are completely dependent on the final target of application. When bioelectronic devices are targeted as in issue (5), issue (1) can be replaced by the following questions. What kinds of proteins and/or enzymes should be selected? How should they be extracted from native bodies? Which part should be modified, and how? Issue (2) then becomes purely chemical (wet) techniques such as the Langmuir–Blodgett (LB) method and the adsorption method in the liquid state rather than physical (dry) methods. The primary purpose of the present paper is to answer these questions.

In biological systems, protein molecules function in molecular recognition, self-organization, photo- and/or electro-activities, and so on. Higher order structures of protein molecules such as the α helix, β sheet and superhelix dominate biological functions. It is quite difficult to grow a large single crystal of protein. Instead, if we can form a two-dimensional (2D) regular array of protein molecules without changing their higher order structure, we then can easily characterize their biological functions and develop so-called bioelectronic devices such as biochips [1], neural networks [2], cellular automata [3] and holographic memories [4]. We have already developed a novel technique [5] combining the LB method with adsorption of protein molecules to a charged polypeptide monolayer and/or to a solid substrate modified chemically.

Bacterial rhodopsins are known as photoactive proteins in *Halobacterium halobium*: a light-driven proton pump, bacteriorhodopsin (BR), a light-driven chloride pump, halorhodopsin (HR), and sensory rhodopsin (SR) and phoborhodopsin (PR), both of which are photoreceptor pigments for the phototaxis of the halobacteria cell. Among these four rhodopsins, BR has been most extensively studied as a model protein for the elucidation of structure and function [6, 7]. BR makes two-dimensional crystals, ordered lattices (purple membrane) in the membrane of *H. halobium*. BR consists of vitamin A aldehyde, retinal, and a protein moiety which contains seven transmembrane α helices. The chromophore retinal is joined to the ε-amino group of a lysine residue (Lys216) by a Schiff base linkage. Isomerization of the retinal in BR from the all-*trans* form to 13-*cis* form is initiated by the absorption of a photon. A series of transient conformational states (photointermediates) then appear within tens of milliseconds. After several photointermediates, BR returns to the orginal state. This cyclic response is called a photoreaction cycle, which is coupled with proton pumping.

Site-specific mutagenesis of BR has been performed [8] to identify several important residues for proton translocation. An aspartic acid residue in position 96 (Asp96) is identified as a proton donor to the Schiff base of the retinal. Asp85 and Asp212 are involved in the proton release phase from the Schiff base. We have tried to find the functionally important residues for ion transport (either proton or chloride) from direct comparison of the amino acid sequences of BR-, HR-, SR-

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and PR-like pigments from newly isolated halobacterial species.

We have recently succeeded in modifying natural protein molecules such as cytochrome b_{562} by means of the one-point and/or two-point mutation technique, in order to enhance the stability, to control the charge distribution and to form a two-dimensional regular packing of protein molecules. The preliminary results of the modification of b_{562} will also be discussed in this paper.

TABLE 1. Presence of four retinal pigments in newly isolated halobacterial species

Species number	BR	HR	SR	PR
1	+ + +	(+)	+	(+)
2	+ +	+ +	+	(+)
3	+ +	+ +	+	(+)
4	+ +	−	−	+
5	+	−	+	+ +

2. Experimental details

2.1. Samples

First, we used ferritin molecules as a typical protein which exhibits non-specific binding to a charged polypeptide monomolecular film (*e.g.* poly-1-benzyl-L-histidine (PBLH)) formed at the air–water interface [5]. Since ferritin is a roughly spherical protein with a diameter of *ca.* 12 nm and contains a core of iron oxide inside the polypeptide shell, the molecular aggregates in two dimensions (monolayers) are observable directly by means of scanning electron microscopy (SEM). Horse spleen ferritin was dissolved in water and fractionated by ultracentrifugation, and the heavier fractions were taken and redissolved in NaCl solution (1–20 mM).

Second, purple membranes of *H. halobium* were prepared according to the standard procedure [9]. Purple membrane fragments were suspended in dimethylformamide to a concentration of 0.5 mg ml^{-1}, spread on the surface of the subphase (containing 0.2–0.4 mM $CaCl_2$), and then transferred onto substrates by a horizontal lift-off method. Several new halobacterial species were isolated from the crude solar salts [10]. The presence of four pigments (BR, HR, SR and PR) was estimated from flash-induced absorbence change measurements and ion pump activity measurements. The presence and relative amounts are indicated by the number of + and − signs in Table 1. The amino acid sequence was deduced from the nucleotide sequence determined by an automated DNA sequencing system (Applied Biosystems model 373A).

Third, *Escherichia coli* overexpressing cytochrome b_{562}, kindly provided by Professor S. Sligar (University of Illinois), was used. Under the conformational estimation with the aid of computer graphics we carried out site-directed (one-point and/or two-point) mutagenesis of native b_{562}. In order to make an LB film of b_{562}, the protohaem IX was extracted from native b_{562}, modified with two alkyl chains as shown in Fig. 1, and then reconstituted.

2.2. Two-dimensional crystallization by the Langmuir–electrostatic adsorption technique

We have developed a novel technique to arrange protein molecules in a well-packed 2D monolayer: the combined technique involving a Langmuir monolayer

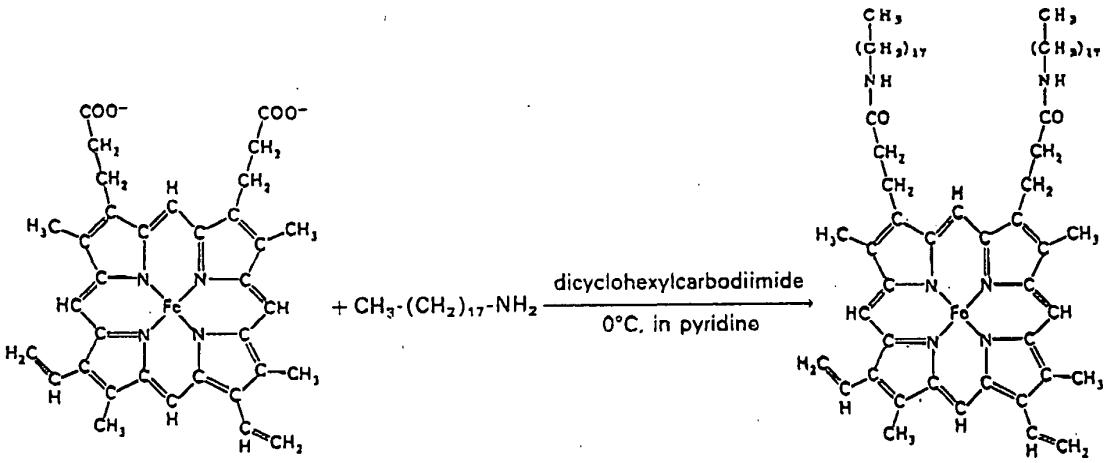


Fig. 1. Modification of the protohaem IX of cytochrome b_{562} with stearylamine.

of charged PBLH and electrostatic adsorption of oppositely charged protein molecules (LA technique). Sufficient PBLH solution (solvent, chloroform containing 0.1% dichloroacetic acid) was spread over the protein solution, *e.g.* ferritin solution in subphase, to ensure an equilibrium surface pressure. The PBLH film was then compressed and kept for several hours. The interfacial film was transferred by a horizontal lift-off technique onto an alkylated silicon wafer. The transferred film was directly observed by SEM (Hitachi S-900) without staining. Direct spreading of protein solution was also available under the optimal subphase conditions (pH, ion strength, etc.), but sometimes denaturation of proteins occurred.

3. Results and discussion

3.1. Two-dimensional packing of ferritin molecules

The monolayer of PBLH at the air–water interface has useful features as follows.

(1) The imidazol group is positively charged under acidic conditions and interactive with acidic proteins in the appropriate pH range.

(2) Because of its polymeric structure, hydrophobic benzyl groups are partly oriented towards the water, which allows us to expect hydrophobic interaction with proteins, stabilizing the binding.

(3) The interfacial film is reversibly compressible owing to its flexibility and stability.

(4) Polymer films are in most cases adequate for horizontal transfer. This is a required condition for avoiding structural disturbances such as rearrangement or overturning, which are serious problems of horizontally transferred lipid monolayers.

(5) The PBLH film is not too rigid, so that fluidity is ensured to some extent.

In the case of ferritin molecules, the LA technique is quite successful in obtaining a well-packed structure. Compression of PBLH monolayer was effective in producing dense packing when the surface density of PBLH was below 1/25 residues $Å^{-2}$. Figure 2 shows an example of an SEM photograph (surface pressure, *ca.* 40 dyn cm^{-1}) [5]. Although there are many defects and/or dislocation-line-like disorder, the ordered part gives higher order optical diffraction patterns (also shown in Fig. 2). It should be noted that if a non-charged polypeptide monolayer (*e.g.* polybenzyl-L-glutamate) is used as an adsorbing Langmuir film, then ferritin molecules cannot be adsorbed onto the monolayer at all, and that by the direct spreading of ferritin molecules at the air–water interface, even under the best subphase conditions, the transferred monolayer has very poor molecular packing, being like coaggregated small islands.

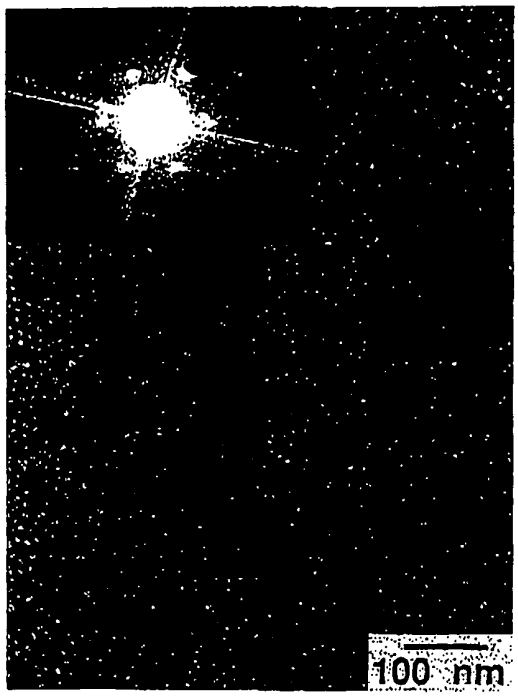


Fig. 2. Scanning electron micrograph of an interfacial film of PBLH and ferritin transferred onto a silicon wafer. The optical diffraction pattern obtained from the dense part of the photograph is also shown.

3.2. Purple membranes [9]

From the observation of directly spread purple membrane monolayer, the following results were obtained: (1) the surface pressure–area curves were time dependent, (2) a spontaneous rise in the surface pressure was observed at high initial coverage, and (3) the thickness and the absorbence at 565 nm for built-up films were smaller than expected. The interfacial denaturation of purple membranes and/or BR molecules was postulated. Structural studies using SEM on the transferred monolayers supported this hypothesis. At a low initial coverage of 25% the size of the purple membrane fragments decreased and their shape became irregular. The coverage ratio also decreased. The substance in the intermembrane space was assayed by sodium dodecylsulphate–polyamide gel electrophoresis to be BR. The result showed that BR denatured from the periphery of the purple membrane fragments and occupied the intermembrane space on the water surface.

More uniform packing could be obtained by increasing the hydrophobic–hydrophilic ratio of the purple membrane fragments. The membrane periphery should have hydrophobic properties, since hydrophobic amino acids of BR are exposed there. To increase the hydrophobicity of purple membrane fragments, they were freeze thawed to be crushed into smaller sizes. Spread-

ing these smaller sized fragments gives a more dense packing.

3.3. Bacteriorhodopsin

To indentify several active-site residues for proton translocation, many kinds of BR point mutants were isolated by the Oesterhelt group [11]. The amino acid changes of all mutants have been determined by their gene sequences. One of the mutants, in which Asp96 was changed to asparagine, has been further characterized. The spectroscopic properties of the BR mutant (Asp96/Asn) in its 2D crystalline purple membrane were investigated. The photoreaction cycle of the mutant (Asp96/Asn) was quite different from that found in the wild type. When the BR mutant (Asp96/Asn) at pH 9 was irradiated by continuous orange light, the visible absorption band quickly disappeared and an absorption band at 410 nm developed. In the light, almost all BR molecules were trapped in an M state. Such a large absorption change was not induced in the wild type under the same solvent and illumination conditions. When the light was turned off, the absorption recovery at 570 nm and decay at 410 nm took place with the same single time constant of about 3 s under physiological conditions. This time constant is *ca.* 1000 times longer than the lifetime of M_{410} in the wild type. The result shows that at this pH the proton uptake was accompanied by the formation of BR (or M_{410} decay). It is suggested that the proton comes directly from the medium to the Schiff base of the retinal.

In clarifying the relation between the amino acid sequence of BR and the photoreaction cycle, site-specific mutagenesis plays an important role. As shown in Table 1 [10], five halobacterial species newly isolated from crude solar salts in our laboratory contain BR-like pigment. We have determined amino acid sequence of these retinal proteins [12]. The sequence of BR-like pigment in five species was different from that of BR of *H. halobium*. However, several amino acid residues around the retinal ("retinal pocket") were conserved in all species (Fig. 3), indicating that these residues are important for the function of BR.

3.4. Cytochrome b_{562}

Cytochrome b_{562} is an electron transport protein with a non-covalently bound protohaem IX prosthetic group, and consists of nearly parallel four-folded α helices [13]. The 2D crystallization of b_{562} by means of the LA technique met with various problems: since p*K* of PBLH is below 7 and p*I* of b_{562} is 7–8, these materials cannot be charged opposite to each other under any pH conditions. Other adsorbing monolayers such as lipids and polypeptides with different electrostatic properties were tried, but failed.

Direct spreading of b_{562} molecules onto a subphase requires some modification of the protein itself: (1) stabilization of higher order structure to avoid denaturation at the air–water interface, (2) the introduction of hydrophobicity, and (3) the modification of surface charge distribution. Item (1) suggests the introduction

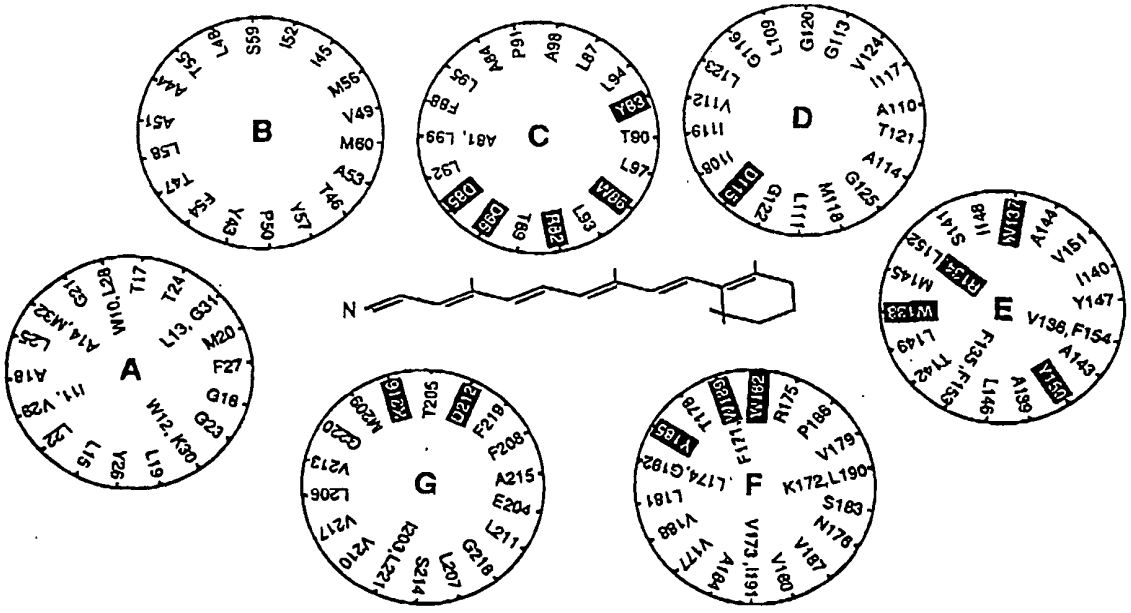
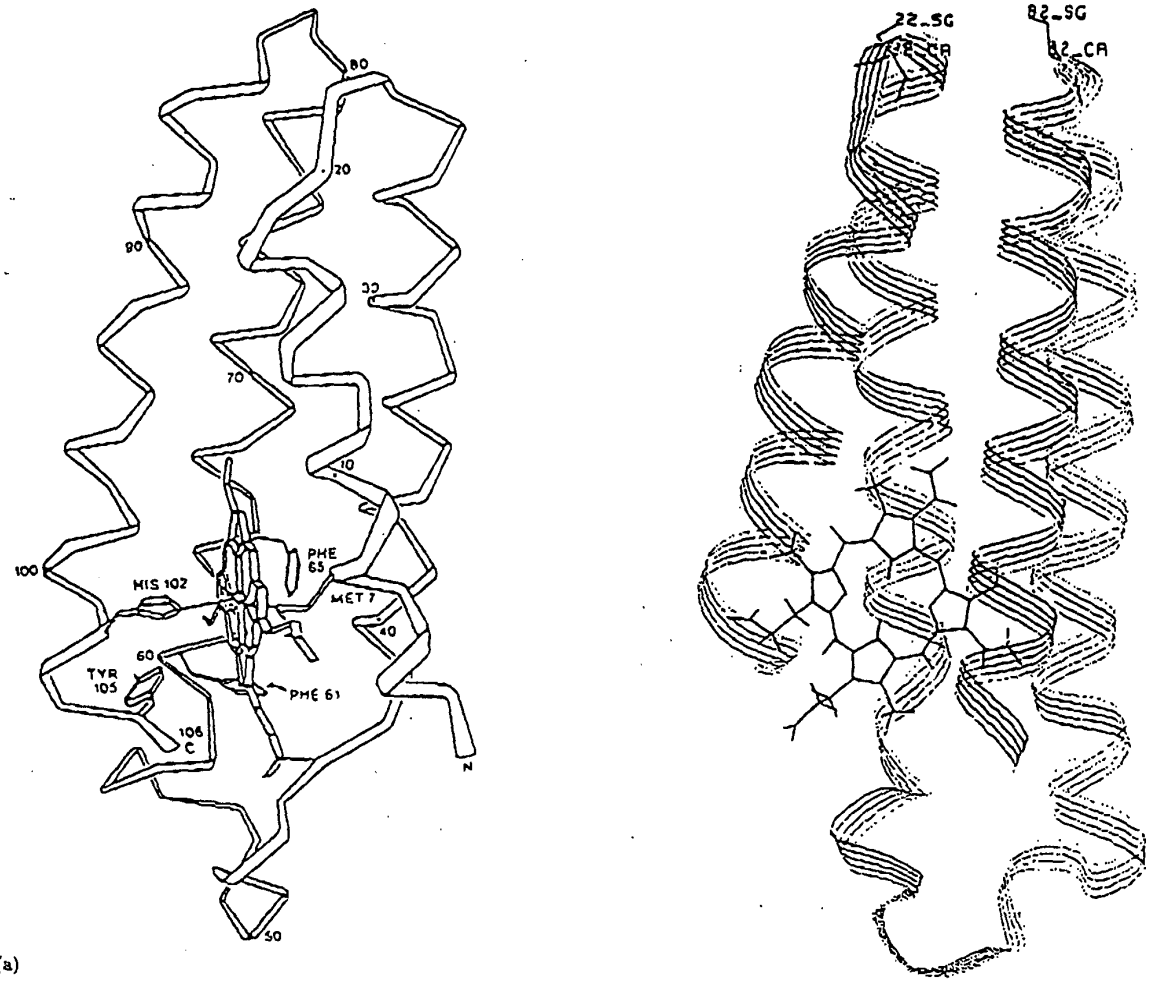


Fig. 3. A helical wheel model of BR. Shaded parts indicate several important residues for the function, which are commonly conserved in all BR-like pigments in newly isolated species.



(a) (b)

Fig. 4. Folded structures of cytochrome b_{562}: (a) proposed model by Weber *et al.* [13] and (b) the refolded structure simulated by CHARMm energy minimization of the 22/88 disulphide mutant b_{562}.

of disulphide bridges between non-helical parts. Following the computer modelling of the mutant by CHARMm, we have estimated the suitable positions to be modified. Figure 4 indicates the CHARMm simulation of energy minimization of the 22/82 disulphide mutant b_{562}, together with a proposed native structure [13]. This suggests the replacement of Asn22 and Gly82 with a cysteine residue which enables the disulphide linkage under the oxidized state to stabilize the wagging of two twin helical columns (A–B and C–D α helices). Details of the modelling theory will be published elsewhere [14].

Site-directed mutagenesis techniques were utilized to produce mutants by introducing a highly specific thiol functional group (cysteine) at two independent points, replacing Asn22 (mutant A22C) and Gly82 (mutant G82C) separately or concurrently (double mutant 22/82C). The thermal stability of these mutants was studied by monitoring the optical absorbence change at 418 nm, which corresponds to the maximum of the Soret band of b_{562}. The result indicated that the wild-type b_{562} denatured thermally around 65 °C, whereas 22/82C was more stable above 70 °C than the wild-type b_{562}. Therefore, the double point mutation is a promising modification for the stabilization of b_{562}.

In the case of item (2), we modified the protohaem IX with stearylamine as shown in Fig. 1; the protohaem has two long alkyl ($-C_{18}H_{37}$) tails (St-protohaem IX). By the insertion of St-protohaem IX into the apoprotein b_{562}, the reconstituted b_{562} (St-b_{562}) acquired hydrophobicity and could form a monolayer at the

air–water interface. The structural analysis of the transferred film is in progress.

4. Conclusion

It has been demonstrated that the LΛ technique (the physical and/or chemical adsorption of molecules in the subphase to the interfacial Langmuir monolayer) is useful for arranging protein molecules in a closely packed 2D array. The importance of genetic modification of native proteins by means of site-directed mutagenesis to enhance the stability of protein during molecular packing and the hydrophobicity was also pointed out.

Acknowledgments

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thin solid films

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